



5 **Aventis Behring GmbH**

**2002/M018 (A66)**

### **Modified cDNA Factor VIII and its Derivatives**

10

The present invention relates to modified DNA sequences coding for biologically active recombinant human factor VIII and its derivatives with improved stability, recombinant expression vectors containing such DNA sequences, host cells transformed with such recombinant expression vectors, and processes for the  
15 manufacture of the recombinant human factor VIII and its derivatives. The invention also covers a transfer vector for use in human gene therapy which comprises such modified DNA sequences.

20

Classic hemophilia or hemophilia A is the most common of the inherited bleeding disorders. It results from a chromosome X-linked deficiency of blood coagulation factor VIII, and affects almost exclusively males with an incidence of between one and two individuals per 10,000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an abnormal bleeding tendency and before treatment with factor  
25 VIII concentrates was introduced the mean life span for a person with severe hemophilia was less than 20 years. The use of concentrates of factor VIII from plasma has considerably improved the situation for the hemophilia patients. The mean life span has increased extensively, giving most of them the possibility to live a more or less normal life. However, there have been certain problems with the  
30 plasma derived concentrates and their use, the most serious of which have been the transmission of viruses. So far, viruses causing AIDS, hepatitis B, and non A non B hepatitis have hit the population seriously. Although different virus inactivation methods and new highly purified factor VIII concentrates have recently been developed an inadvertant contamination can not be excluded. Also, the factor

- 5 VIII concentrates are fairly expensive because of the limited supply of human plasma raw material.

A factor VIII product derived from recombinant material is likely to solve a large extent of the problems associated with the use of plasma derived factor VIII  
10 concentrates for treatment for hemophilia A. However, the development of a recombinant factor VIII has met some difficulties, for instance the problem of achieving production levels in sufficiently high yields, in particular regarding the full-length molecule.

- 15 In fresh plasma prepared in the presence of protease inhibitors, factor VIII has been shown to have a molecular weight of 280 kDa and to be composed of two polypeptide chains of 200 kDa and 80 kDa, respectively (Andersson, L.-O., et al. (1986) Proc. Natl. Acad. Sci. USA 83, 2979-2983). These chains are held together by metal ion bridges. More or less proteolytically degraded forms of the factor VIII  
20 molecule can be found as active fragments in factor VIII material purified from commercial concentrates (Andersson, L.-O., et al. ibid.; Andersson, L.-O., et al. (1985) EP 0 197 901). The fragmented form of factor VIII having molecular weights from 260 kDa down to 170 kDa, consists of one heavy chain with a molecular weight ranging from 180 kDa down to 90 kDa, where all variants have identical  
25 amino termini, in combination with one 80 kDa light chain. The amino-terminal region of the heavy chain is identical to that of the single chain factor VIII polypeptide that can be deduced from the nucleotide sequence data of the factor VIII cDNA (Wood, W.I., et al. (1984) Nature 312, 330-336; Vehar, G.A., et al. (1984) Nature 312, 337-342).

30

- The smallest active form of factor VIII with a molecular weight of 170 kDa, consisting of one 90 kDa and one 80 kDa chain, can be activated with thrombin to the same extent as the higher molecular weight forms, and thus represents an unactivated form. It has also been shown to have full biological activity in vivo as  
35 tested in hemophilia dogs (Brinkhous, K.M., et al. (1985) Proc. Natl. Acad. Sci. USA

5 82, 8752-8756). Thus, the haemostatic effectiveness of the 170 kDa form is the same as for the high molecular weight forms of factor VIII.

The fact that the middle heavily glycosylated region of the factor VIII polypeptide chain residing between amino acids Arg-740 and Glu-1649 does not seem to be  
10 necessary for full biological activity has prompted several researchers to attempt to produce derivatives of recombinant factor VIII lacking this region. This has been achieved by deleting a portion of the cDNA encoding the middle heavily glycosylated region of factor VIII either entirely or partially.

15 For example, J.J. Toole, et al, reported the construction and expression of factor VIII lacking amino acids 982 through 1562, and 760 through 1639 respectively (Proc. Natl. Acad. Sci. USA (1986) 83, 5939-5942). D.L. Eaton, et al. reported the construction and expression of factor VIII lacking amino acids 797 through 1562 (Biochemistry (1986) 25, 8343-8347). R.J. Kaufman described the expression of  
20 factor VIII lacking amino acids 741 through 1646 (PCT application No. WO 87/04187). N. Sarver, et al. reported the construction and expression of factor VIII lacking amino acids 747 through 1560 (DNA (1987) 6, 553-564). M. Pasek reported the construction and expression of factor VIII lacking amino acids 745 through 1562, and amino acids 741 through 1648, respectively (PCT application No. WO  
25 88/00831). K.-D. Langner reported the construction and expression of factor VIII lacking amino acids 816 through 1598, and amino acids 741 through 1689, respectively (Behring Inst. Mitt., (1988) No. 82, 16-25, EP 0 295 597). P. Meulien, et al., reported the construction and expression of factor VIII lacking amino acids 868 through 1562, and amino acids 771 through 1666, respectively (Protein Engineering  
30 (1988) 2(4), 301-306, EP 0 303 540). When expressing these deleted forms of factor VIII cDNA in mammalian cells the production level is typically 10 times higher as compared to full-length factor VIII.

FVIII is secreted into plasma as a heterodimer of a heavy chain (domains A1-A2-B)  
35 and a light chain (A3-C1-C2) associated through a noncovalent divalent metal ion

5 linkage between the A1- and A3 domains. In plasma, FVIII is stabilized by binding to von Willebrand factor.

10 Upon proteolytic activation by thrombin, FVIII is activated to a heterotrimer of 2 heavy chain fragments (A1, a 50 kDa fragment, and A2 a 43 kDa fragment) and the light chain (A3-C1-C2, a 73 kDa fragment). The active form of FVIII (FVIIIa) thus consists of an A1-subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit associated with the A1 domain . The dissociation of that free A2 subunit from the heterotrimer is thought to be the rate limiting step in FVIIIa inactivation after thrombin activation  
15 (Fay, P.J. et al, J. Biol. Chem. 266: 8957 (1991), Fay PJ & Smudzin TM, J. Biol. Chem. 267: 13246-50 (1992)). The half life of FVIIIa in plasma is only 2.1 minutes (Saenko et al., Vox Sang. 83: 89-96 (2002)). To enhance the half life of FVIIIa would result into a longer acting FVIIIa which would also translate into less frequent injections of such a FVIII preparation. The inactivation of FVIIIa through  
20 activated Protein C (APC) by cleavage at Arg336 and Arg562 is thought not to be the rate limiting step. Attempts have been made to create a FVIIIa which is inactivation resistant by covalently attaching the A2 domain to the A3 domain and by mutating the APC cleavage sites (Pipe and Kaufman, PNAS, 94:11851-11856). However such a FVIIIa could have a thrombogenic potential as it is almost  
25 completely inactivation resistant. It is therefore the purpose of this invention to create a FVIIIa in which the A2 domain is stabilized without completely blocking inactivation.

30 FVIII is administered i.v. to haemophilia patients who are on prophylactic treatment about 3 times per week due to the plasma half life of FVIII of about 12 hours. It would thus be highly desirable to create a FVIII with enhanced plasma half life which could lead to a FVIII preparation which has to be administered less frequently. The present invention offers a solution to this problem by a modified FVIII molecule with an increased association of A2 to the A1/A3-C1-C2.

5 The nature of these modifications was identified by comparing the sequence of porcine FVIII to that of human FVIII as it is known that the dissociation of human A2 domain is threefold enhanced versus that of porcine A2 (Lollar et al., J Biol. Chem., 267:23652-23657 (1992)). The sequence comparison (Fig. 1) revealed several differences. A subset of these differences consists of differently charged amino  
10 acids. Mutants of human FVIII were constructed according to the following guidelines. When the human sequence contained a neutral amino acid whereas the porcine sequence contained a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence was introduced into the human sequence. When the human sequence contained a charged amino acid whereas  
15 the porcine FVIII contained a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge was introduced, e.g. if the human FVIII contained an acidic amino acid at a position where the porcine FVIII contained a neutral amino acid, also a basic amino acid was introduced. When the human sequence contained a charged sequence whereas the porcine FVIII contained a charged  
20 amino acid then an amino acid with the same charge as found in the porcine amino acid was introduced into the human sequence. Examples for such mutations which lead to an improved FVIII with a plasma half life of its activated form of more than three minutes, preferably of more than 10 minutes even more preferably more than 30 minutes, are listed in figure 2.

25 Other mutations for an improved FVIII were deduced by analyzing mutations in human FVIII which occurred naturally and which lead to a faster dissociation of the A2 domain associated with hemophilia. Such mutations result in differences between the two-stage assay as compared to the one-stage assay while  
30 determining FVIII clotting activity, whereas the two-stage assay result is lower than that of the one-stage assay as in the two-stage assay an incubation time of several minutes allows an unstable A2 domain to dissociate (Saenko et al., Vox Sang., 83:89-96 (2002). It was inferred that in those cases where such an increased instability was the result of the introduction of a charged amino acid that amino acid  
35 should be mutated into one of the opposite charge. Examples for such mutations

- 5    which lead to an improved FVIII with a plasma half life of its activated form of more than three minutes, preferably of more than 10 minutes, even more preferably more than 30 minutes, are listed in figure 3.

10    As a basis for introducing the mutations preferably a modified factor VIII cDNA is used which comprises a first DNA segment coding for the amino acids 1 through 740 of the human factor VIII and a second DNA segment coding for the amino acids 1649 through 2332 of the human factor VIII. These two segments may be interconnected by a linker DNA segment, but the invention also encompasses introducing the mutations into full length FVIII.

15

Subject of the invention is therefore a modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment, and

20

A)    one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA are substituted by a different codon in such a way that

25

- when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30

- when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the opposite charge is introduced into the human sequence

- 5           • when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino acid with the opposite charge then a codon for an amino acid with the opposite charge is introduced into the human sequence or

- 10   B)    one or several codons for a charged amino acid which are found in the FVIII cDNA of a hemophilic patient are replaced by a codon for an amino acid of the opposite charge.

The production of factor VIII proteins at high levels in suitable host cells, requires  
15 the assembly of the above-mentioned modified factor VIII DNA's into efficient transcriptional units together with suitable regulatory elements in a recombinant expression vector, that can be propagated in E. coli according to methods known to those skilled in the art. Efficient transcriptional regulatory elements could be derived from viruses having animal cells as their natural hosts or from the chromosomal  
20 DNA of animal cells. Preferably, promoter-enhancer combinations derived from the Simian Virus 40, adenovirus, BK polyoma virus, human cytomegalovirus, or the long terminal repeat of Rous sarcoma virus, or promoter-enhancer combinations including strongly constitutively transcribed genes in animal cells like beta-actin or GRP78 can be used. In order to achieve stable high levels of mRNA transcribed  
25 from the factor VIII DNA's, the transcriptional unit should contain in its 3'-proximal part a DNA region encoding a transcriptional termination-polyadenylation sequence. Preferably, this sequence is derived from the Simian Virus 40 early transcriptional region, the rabbit beta-globin gene, or the human tissue plasminogen activator gene.

30

The factor VIII cDNA's are then integrated into the genome into a suitable host cell line for expression of the factor VIII proteins. Preferably this cell line should be an animal cell-line of vertebrate origin in order to ensure correct folding, disulfide bond formation, asparagines-linked glycosylation and other post-translational  
35 modifications as well as secretion into the cultivation medium. Examples on other

5 post-translational modifications are tyrosine O-sulfation, and proteolytic processing of the nascent polypeptide chain. Examples of cell lines that can be use are monkey COS-cells, mouse L-cells, mouse C127-cells, hamster BHK-21 cells, human embryonic kidney 293 cells, and preferentially CHO-cells.

10 The recombinant expression vector encoding the factor VIII cDNA's can be introduced into an animal cell line in several different ways. For instance, recombinant expression vectors can be created from vectors based on different animal viruses, Examples of these are vectors based on baculovirus, vaccinia virus, adenovirus, and preferably bovine papilloma virus.

15

The transcription units encoding the factor VIII DNA's can also be introduced into animal cells together with another recombinant gene which may function as a dominant selectable marker in these cells in order to facilitate the isolation of specific cell clones which have integrated the recombinant DNA into their genome.

20 Examples of this type of dominant selectable marker genes are Tn5 aminoglycoside phosphotransferase, conferring resistance to Geneticin (G418), hygromycin phosphotransferase, conferring resistance to hygromycin, and puromycin acetyl transferase, conferring resistance to puromycin. The recombinant expression vector encoding such a selectable marker can reside either on the same vector as the one  
25 encoding the factor VIII cDNA, or it can be encoded on a separate vector which is simultaneously introduced and integrated to the genome of the host cell, frequently resulting in a tight physical linkage between the different transcription units.

Other types of selectable marker genes which can be used together with the factor  
30 VIII DNA's are based on various transcription units encoding dihydrofolate reductase (dhfr). After introduction of this type of gene into cells lacking endogenous dhfr-activity, preferentially CHO-cells (DUKX-B11, DG-44) it will enable these to grow in media lacking nucleosides. An example of such a medium is Ham's F12 without hypoxanthin, thymidin, and glycine. These dhfr-genes can be  
35 introduced together with the factor VIII cDNA transcriptional units into CHO-cells of

5 the above type, either linked on the same vector or on different vectors, thus creating dhfr-positive cell lines producing recombinant factor VIII protein.

If the above cell lines are grown in the presence of the cytotoxic dhfr-inhibitor methotrexate, new cell lines resistant to methotrexate will emerge. These cell lines  
10 may produce recombinant factor VIII protein at an increased rate due to the amplified number of linked dhfr and factor VIII transcriptional units. When propagating these cell lines in increasing concentrations of methotrexate (1-10000 nM), new cell lines can be obtained which produce factor VIII protein at very high rate.

15

The above cell lines producing factor VIII protein can be grown on a large scale, either in suspension culture or on various solid supports. Examples of these supports are microcarriers based on dextran or collagen matrices, or solid supports in the form of hollow fibres or various ceramic materials. When grown in cell  
20 suspension culture or on microcarriers the culture of the above cell lines can be performed either as a bath culture or as a perfusion culture with continuous production of conditioned medium over extended periods of time. Thus, according to the present invention, the above cell lines are well suited for the development of an industrial process for the production of recombinant factor VIII that can be  
25 isolated from human plasma.

The recombinant factor VIII protein which accumulate in the medium of CHO-cells of the above type, can be concentrated and purified by a variety of biochemical and chromatographic methods, including methods utilizing differences in size, charge,  
30 hydrophobicity, solubility, specific affinity, etc. between the recombinant factor VIII protein and other substances in the cell cultivation medium.

An example of such a purification is the adsorption of the recombinant factor VIII protein to a monoclonal antibody which is immobilised on a solid support. After

5 desorption, the factor VIII protein can be further purified by a variety of chromatographic techniques based on the above properties.

The recombinant proteins with factor VIII activity described in this invention can be formulated into pharmaceutical preparations for therapeutic use. The purified factor  
10 VIII proteins may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical adjuvants to provide pharmaceutical preparations.

The modified factor VIII DNA's of this invention may also be integrated into a  
15 transfer vector for use in the human gene therapy.

A further subject of this invention is a modified biologically active recombinant human factor VIII with improved plasma half life of its activated form wherein mutations are inserted either in the wild-type factor VIII or in a FVIII in which the B-  
20 domain is partially or completely deleted and replaced by a linker, and

A) one or several amino acids of the human factor VIII which are not identical with the corresponding amino acid in the same position of the porcine factor VIII are substituted by a different amino acid in such a way that

25 • when the human sequence contains a neutral amino acid whereas the porcine sequence contains a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30 • when the human sequence contains a charged amino acid whereas the porcine sequence contains a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge is introduced into the human sequence;

- 5                   • when the human sequence contains a charged amino acid  
                      whereas the porcine sequence contains an amino acid with the  
                      opposite charge then an amino acid with the opposite charge is  
                      introduced into the human sequence or

- 10    B)    one or several charged amino acids which are found in the FVIII amino  
          sequence of hemophilic patients are replaced by a codon for an amino acid of the  
          opposite charge.

15    The present invention will be further described more in detail in the following  
      examples thereof. This description of specific embodiments of the invention will be  
      made in conjunction with the appended figures.

#### **Generation of FVIII mutants**

20    For the generation of FVIII mutants, a suitable subfragment of the FVIII cDNA (e.g.  
      Aval - SacI, encompassing aminoacids 226 to 978) is first subcloned into a suitable  
      cloning vector to reduce subsequent sequencing efforts. Site directed mutagenesis  
      is then performed with a commercially available mutagenesis kit (e.g. QuickChange  
      SiteDirected Mutagenesis Kit (Stratagene) according to the manufacturer's  
      instructions. Primers used for mutagenesis are listed in the attached sequence  
25    listing and below, where the mutagenic bases are indicated in bold letters.

Mutation:           A284K

Forward primer

5'GGAACCATCGCCAG**A**AGTCCTTGGAAATCTCGCC<sup>3'</sup>    (Sequence 1)

30    Reverse primer

5'GGCGAGATT**T**CCAAGGACTTCTGGCGATGGTTCC<sup>3'</sup>    (Sequence 2)

Mutation:    D318G

Forward primer

35    5'CCCACCAACATGG**T**GGCATGGAAGCTTATGTC<sup>3'</sup>    (Sequence 3)

5 Reverse primer

5'GACATAAGCTTCCATGCC**AC**CATGTTGGTGGG<sup>3'</sup> (Sequence 4)

Mutation: M337R

Forward primer

10 5'CAGAGGAACCCCAACTACGAC**CG**TAAAAATAATGAAGAAGCGGAAGAC<sup>3'</sup>  
(Sequence 5)

Reverse primer

5'GTCTTCCGCTTCTTCATTATTTTT**AC**GTCGTAGTTGGGGTTCCTCTG<sup>3'</sup>  
(Sequence 6)

15

Mutation: N340D

Forward primer

5'CCCAACTACGAATGAAAAAT**G**ATGAAGAAGCGGAAGACTATG<sup>3'</sup>  
(Sequence 7)

20 Reverse primer

5'CATAGTCTTCCGCTTCTTCAT**C**ATTTTTTCATTCGTAGTTGGG<sup>3'</sup>  
(Sequence 8)

Mutation: D349N

25 Forward primer

5'GAAGAAGCGGAAGACTATGATGATA**A**ATCTTACTGATTCTG<sup>3'</sup>  
(Sequence 9)

Reverse primer 5'CAGAATCAGTAAGATTATCATCATAGTCTTCCGCTTCTTC<sup>3'</sup>  
(Sequence 10)

30

Mutation: N364D

Forward primer 5'GGTCAGGTTTGATGATGAC**G**ACTCTCCTTCCTTTATCC<sup>3'</sup>  
(Sequence 11)

Reverse primer 5'GGATAAAGGAAGGAGAGT**C**GTCATCATCAAACCTGACC<sup>3'</sup>  
(Sequence 12)

35

5

Mutation: D403S

Forward primer 5'CCCTTAGTCCTCGCCCCCTCTGACAGAAGTTATAAAAG<sup>3'</sup>  
(Sequence 13)

Reverse primer 5'CTTTTATAACTTCTGTCAGAGGGGGCGAGGACTAAGGG<sup>3'</sup>  
(Sequence 14)

10

Mutation: E434V

Forward primer  
5'GTCCGATTTATGGCATAACAGATGTTACCTTTAAGACTCG<sup>3'</sup>  
(Sequence 15)

15

Reverse primer  
5'CGAGTCTTAAAGGTAAACATCTGTGTATGCCATAAATCGGAC<sup>3'</sup>  
(Sequence 16)

20

Mutation: E440K

Forward primer  
5'CCTTTAAGACTCGTAAAGCTATTCAGCATGAATCAGG<sup>3'</sup> (Sequence 17)

Reverse primer

5'CCTGATTCATGCTGAATAGCTTTACGAGTCTTAAAGG<sup>3'</sup> (Sequence 18)

25

Mutation: Q468K

Forward primer  
5'CACACTGTTGATTATATTTAAGAATAAAGCAAGCAGACCATATAAC<sup>3'</sup>  
(Sequence 19)

30

Reverse primer  
5'GTTATATGGTCTGCTTGCTTTATTCTTAAATATAATCAACAGTGTG<sup>3'</sup>  
(Sequence 20)

35

Mutation: R484S

5 Forward primer  
5'CCCTCACGGAATCACTGATGTCTCTCCTTTGTATTCAAGG<sup>3'</sup>  
(Sequence 21)

Reverse primer  
5'CCTTGAATACAAAGGAGAGACATCAGTGATTCCGTGAGGG<sup>3'</sup>  
10 (Sequence 22)

Mutation: R489G

Forward primer 5'GATGTCCGTCCTTTGTATTCAGGGAGATTACCAAAGG<sup>3'</sup>  
(Sequence 23)

15 Reverse primer 5'CCTTTTGGTAATCTCCCTGAATACAAAGGACGGACATC<sup>3'</sup>  
(Sequence 24)

Mutation: R583Q

Forward primer

20 5'CTGTATTTGATGAGAACC~~AA~~AGCTGGTACCTCACAG<sup>3'</sup> (Sequence 25)

Reverse primer

5'CTGTGAGGTACCAGCTTTGGTTCTCATCAAATACAG<sup>3'</sup> (Sequence 26)

Mutation: A599D

25 Forward primer

5'CTCCCCAATCCAGATGGAGTGCAGCTTGAG<sup>3'</sup> (Sequence 27)

Reverse primer

5'CTCAAGCTGCACTCCATCTGGATTGGGGAG<sup>3'</sup> (Sequence 28)

30 Mutation: E604Q

Forward primer

5'CAGCTGGAGTGCAGCTT~~C~~AGGATCCAGAGTTC<sup>3'</sup> (Sequence 29)

Reverse primer

5'GA~~A~~CTCTGGATCCTGAAGCTGCACTCCAGCTG<sup>3'</sup> (Sequence 30)

35

5 Mutation: G1948K

Forward primer

5'CGATGGTATCTGCTCAGCATGA**AAG**AGCAATGAAAACATCCATTCTATT

C<sup>3'</sup> (Sequence 31)

Reverse primer

10 5'GAATAGAATGGATGTTTTATTGCT**CTT**CATGCTGAGCAGATACCATCG

3' (Sequence 32)

After clone isolation and sequence verification mutant subfragments are reinserted into the respective expression vector.

15

### **Expression of FVIII mutants**

Transfection of FVIII mutant clones and expression of the mutant FVIII molecules is done as described previously and known to those skilled in the art (e.g. Plantier JL et al. Thromb. Haemost. 86:596-603 (2001)).

20

### **Measuring affinity of A2 subunit for A1/A3-C1-C2**

The increased affinity of the A2 subunit for the A1/A3-C1-C2 can be measured as previously described by functional assays (Fay PJ & Smudzin TM. J. Biol. Chem. 267:13246-50 (1992); Lollar P et al. J. Biol. Chem. 267:23652-57 (1992)) as well as  
25 a physical assay employing surface plasmon resonance (Persson E et al. Biochemistry 34:12775-81 (1995)).

The sequence of the porcine factor VIII is shown in Sequence 33, whereas the sequence of the human factor VIII is shown in sequence 34 of the attached  
30 sequence listing.

In the following Sequence Listing Sequences 1-32 describe oligonucleotides which are used to introduce specific mutations into FVIII. Sequence 33 is the amino acid sequence of full length mature porcine FVIII, Sequence 34 is the amino acid  
35 sequence of full length mature human FVIII.



SEQUENCE LISTING

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<130> 2002/M018-A66

<140> US/10/721,997

<141> 2003-11-26

<160> 34

<170> PatentIn version 3.1

<210> 1

<211> 34

<212> DNA

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34

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<221> Reverse primer

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34

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Ala	Thr	Ala	Pro	Gly	Ala	Leu	Pro	Leu	Gly	Pro	Ser	Val	Leu	Tyr	Lys
		35					40					45			

Lys	Thr	Val	Phe	Val	Glu	Phe	Thr	Asp	Gln	Leu	Phe	Ser	Val	Ala	Arg
50						55					60				

Pro	Arg	Pro	Pro	Trp	Met	Gly	Leu	Leu	Gly	Pro	Thr	Ile	Gln	Ala	Glu
65					70					75					80

Val	Tyr	Asp	Thr	Val	Val	Val	Thr	Leu	Lys	Asn	Met	Ala	Ser	His	Pro
				85					90					95	

Val	Ser	Leu	His	Ala	Val	Gly	Val	Ser	Phe	Trp	Lys	Ser	Ser	Glu	Gly
			100					105					110		

Ala	Glu	Tyr	Glu	Asp	His	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	Asp	Lys
		115					120					125			

Val	Leu	Pro	Gly	Lys	Ser	Gln	Thr	Tyr	Val	Trp	Gln	Val	Leu	Lys	Glu
	130					135					140				

Asn Gly Pro Thr Ala Ser Asp Pro Pro Cys Leu Thr Tyr Ser Tyr Leu  
145 150 155 160

Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala  
165 170 175

Leu Leu Val Cys Arg Glu Gly Ser Leu Thr Arg Glu Arg Thr Gln Asn  
180 185 190

Leu His Glu Phe Val Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser  
195 200 205

Trp His Ser Ala Arg Asn Asp Ser Trp Thr Arg Ala Met Asp Pro Ala  
210 215 220

Pro Ala Arg Ala Gln Pro Ala Met His Thr Val Asn Gly Tyr Val Asn  
225 230 235 240

Arg Ser Leu Pro Gly Leu Ile Gly Cys His Lys Lys Ser Val Tyr Trp  
245 250 255

His Val Ile Gly Met Gly Thr Ser Pro Glu Val His Ser Ile Phe Leu  
260 265 270

Glu Gly His Thr Phe Leu Val Arg His His Arg Gln Ala Ser Leu Glu  
275 280 285

Ile Ser Pro Leu Thr Phe Leu Thr Ala Gln Thr Phe Leu Met Asp Leu  
290 295 300

Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His His His Gly Gly  
305 310 315 320

Met Glu Ala His Val Arg Val Glu Ser Cys Ala Glu Glu Pro Gln Leu  
325 330 335

Arg Arg Lys Ala Asp Glu Glu Glu Asp Tyr Asp Asp Asn Leu Tyr Asp  
340 345 350

Ser Asp Met Asp Val Val Arg Leu Asp Gly Asp Asp Val Ser Pro Phe  
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His  
370 375 380

Tyr Ile Ser Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Ala Val Pro  
385 390 395 400

Ser Pro Ser Asp Arg Ser Tyr Lys Ser Leu Tyr Leu Asn Ser Gly Pro  
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Ala Arg Phe Val Ala Tyr Thr  
420 425 430

Asp Val Thr Phe Lys Thr Arg Lys Ala Ile Pro Tyr Glu Ser Gly Ile  
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile  
450 455 460

Phe Lys Asn Lys Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile  
465 470 475 480

Thr Asp Val Ser Ala Leu His Pro Gly Arg Leu Leu Lys Gly Trp Lys  
485 490 495

His Leu Lys Asp Met Pro Ile Leu Pro Gly Glu Thr Phe Lys Tyr Lys  
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys  
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Ser Ile Asn Leu Glu Lys Asp Leu Ala  
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp  
545 550 555 560

Gln Arg Gly Asn Gln Met Met Ser Asp Lys Arg Asn Val Ile Leu Phe  
565 570 575

Ser Val Phe Asp Glu Asn Gln Ser Trp Tyr Leu Ala Glu Asn Ile Gln  
580 585 590

Arg Phe Leu Pro Asn Pro Asp Gly Leu Gln Pro Gln Asp Pro Glu Phe  
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser  
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu  
625 630 635 640

Ser Val Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr  
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro  
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp  
675 680 685

Val Leu Gly Cys His Asn Ser Asp Leu Arg Asn Arg Gly Met Thr Ala  
690 695 700

Leu Leu Lys Val Tyr Ser Cys Asp Arg Asp Ile Gly Asp Tyr Tyr Asp  
705 710 715 720

Asn Thr Tyr Glu Asp Ile Pro Gly Phe Leu Leu Ser Gly Lys Asn Val  
725 730 735

Ile Glu Pro Arg Ser Phe Ala Gln Asn Ser Arg Pro Pro Ser Ala Ser  
740 745 750

Gln Lys Gln Phe Gln Thr Ile Thr Ser Pro Glu Asp Asp Val Glu Leu  
755 760 765

Asp Pro Gln Ser Gly Glu Arg Thr Gln Ala Leu Glu Glu Leu Ser Val  
770 775 780

Pro Ser Gly Asp Gly Ser Met Leu Leu Gly Gln Asn Pro Ala Pro His  
785 790 795 800

Gly Ser Ser Ser Ser Asp Leu Gln Glu Ala Arg Asn Glu Ala Asp Asp

805										810					815				
Tyr	Leu	Pro	Gly	Ala	Arg	Glu	Arg	Asn	Thr	Ala	Pro	Ser	Ala	Ala	Ala				
			820					825					830						
Arg	Leu	Arg	Pro	Glu	Leu	His	His	Ser	Ala	Glu	Arg	Val	Leu	Thr	Pro				
		835					840					845							
Glu	Pro	Glu	Lys	Glu	Leu	Lys	Lys	Leu	Asp	Ser	Lys	Met	Ser	Ser	Ser				
	850					855					860								
Ser	Asp	Leu	Leu	Lys	Thr	Ser	Pro	Thr	Ile	Pro	Ser	Asp	Thr	Leu	Ser				
865					870					875					880				
Ala	Glu	Thr	Glu	Arg	Thr	His	Ser	Leu	Gly	Pro	Pro	His	Pro	Gln	Val				
				885					890					895					
Asn	Phe	Arg	Ser	Gln	Leu	Gly	Ala	Ile	Val	Leu	Gly	Lys	Asn	Ser	Ser				
			900					905					910						
His	Phe	Ile	Gly	Ala	Gly	Val	Pro	Leu	Gly	Ser	Thr	Glu	Glu	Asp	His				
		915					920					925							
Glu	Ser	Ser	Leu	Gly	Glu	Asn	Val	Ser	Pro	Val	Glu	Ser	Asp	Gly	Ile				
	930					935					940								
Phe	Glu	Lys	Glu	Arg	Ala	His	Gly	Pro	Ala	Ser	Leu	Thr	Lys	Asp	Asp				
945					950					955					960				
Val	Leu	Phe	Lys	Val	Asn	Ile	Ser	Leu	Val	Lys	Thr	Asn	Lys	Ala	Arg				
				965					970					975					
Val	Tyr	Leu	Lys	Thr	Asn	Arg	Lys	Ile	His	Ile	Asp	Asp	Ala	Ala	Leu				
			980					985					990						
Leu	Thr	Glu	Asn	Arg	Ala	Ser	Ala	Thr	Phe	Met	Asp	Lys	Asn	Thr	Thr				
		995					1000					1005							
Ala	Ser	Gly	Leu	Asn	His	Val	Ser	Asn	Trp	Ile	Lys	Gly	Pro	Leu					
	1010					1015					1020								

Gly	Lys	Asn	Pro	Leu	Ser	Ser	Glu	Arg	Gly	Pro	Ser	Pro	Glu	Leu
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Leu	Thr	Ser	Ser	Gly	Ser	Gly	Lys	Ser	Val	Lys	Gly	Gln	Ser	Ser
1040						1045					1050			
Gly	Gln	Gly	Arg	Ile	Arg	Val	Ala	Val	Glu	Glu	Glu	Glu	Leu	Ser
1055						1060					1065			
Lys	Gly	Lys	Glu	Met	Met	Leu	Pro	Asn	Ser	Glu	Leu	Thr	Phe	Leu
1070						1075					1080			
Thr	Asn	Ser	Ala	Asp	Val	Gln	Gly	Asn	Asp	Thr	His	Ser	Gln	Gly
1085						1090					1095			
Lys	Lys	Ser	Arg	Glu	Glu	Met	Glu	Arg	Arg	Glu	Lys	Leu	Val	Gln
1100						1105					1110			
Glu	Lys	Val	Asp	Leu	Pro	Gln	Val	Tyr	Thr	Ala	Thr	Gly	Thr	Lys
1115						1120					1125			
Asn	Phe	Leu	Arg	Asn	Ile	Phe	His	Gln	Ser	Thr	Glu	Pro	Ser	Val
1130						1135					1140			
Glu	Gly	Phe	Asp	Gly	Gly	Ser	His	Ala	Pro	Val	Pro	Gln	Asp	Ser
1145						1150					1155			
Arg	Ser	Leu	Asn	Asp	Ser	Ala	Glu	Arg	Ala	Glu	Thr	His	Ile	Ala
1160						1165					1170			
His	Phe	Ser	Ala	Ile	Arg	Glu	Glu	Ala	Pro	Leu	Glu	Ala	Pro	Gly
1175						1180					1185			
Asn	Arg	Thr	Gly	Pro	Gly	Pro	Arg	Ser	Ala	Val	Pro	Arg	Arg	Val
1190						1195					1200			
Lys	Gln	Ser	Leu	Lys	Gln	Ile	Arg	Leu	Pro	Leu	Glu	Glu	Ile	Lys
1205						1210					1215			
Pro	Glu	Arg	Gly	Val	Val	Leu	Asn	Ala	Thr	Ser	Thr	Arg	Trp	Ser
1220						1225					1230			

Glu	Ser	Ser	Pro	Ile	Leu	Gln	Gly	Ala	Lys	Arg	Asn	Asn	Leu	Ser
1235						1240					1245			
Leu	Pro	Phe	Leu	Thr	Leu	Glu	Met	Ala	Gly	Gly	Gln	Gly	Lys	Ile
1250						1255					1260			
Ser	Ala	Leu	Gly	Lys	Ser	Ala	Ala	Gly	Pro	Leu	Ala	Ser	Gly	Lys
1265						1270					1275			
Leu	Glu	Lys	Ala	Val	Leu	Ser	Ser	Ala	Gly	Leu	Ser	Glu	Ala	Ser
1280						1285					1290			
Gly	Lys	Ala	Glu	Phe	Leu	Pro	Lys	Val	Arg	Val	His	Arg	Glu	Asp
1295						1300					1305			
Leu	Leu	Pro	Gln	Lys	Thr	Ser	Asn	Val	Ser	Cys	Ala	His	Gly	Asp
1310						1315					1320			
Leu	Gly	Gln	Glu	Ile	Phe	Leu	Gln	Lys	Thr	Arg	Gly	Pro	Val	Asn
1325						1330					1335			
Leu	Asn	Lys	Val	Asn	Arg	Pro	Gly	Arg	Thr	Pro	Ser	Lys	Leu	Leu
1340						1345					1350			
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1355						1360					1365			
Lys	Ser	Thr	Ala	Leu	Arg	Thr	Lys	Asp	Ile	Ile	Ser	Leu	Pro	Leu
1370						1375					1380			
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1385						1390					1395			
Gln	Ala	Glu	Thr	Gln	Arg	Glu	Ala	Ala	Trp	Thr	Lys	Gln	Gly	Gly
1400						1405					1410			
Pro	Gly	Arg	Leu	Cys	Ala	Pro	Lys	Pro	Pro	Val	Leu	Arg	Arg	His
1415						1420					1425			
Gln	Arg	Asp	Ile	Ser	Leu	Pro	Thr	Phe	Gln	Pro	Glu	Glu	Asp	Lys
1430						1435					1440			

Met	Asp	Tyr	Asp	Asp	Ile	Phe	Ser	Thr	Glu	Thr	Lys	Gly	Glu	Asp
1445						1450					1455			
Phe	Asp	Ile	Tyr	Gly	Glu	Asp	Glu	Asn	Gln	Asp	Pro	Arg	Ser	Phe
1460						1465					1470			
Gln	Lys	Arg	Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val	Glu	Gln	Leu
1475						1480					1485			
Trp	Asp	Tyr	Gly	Met	Ser	Glu	Ser	Pro	Arg	Ala	Leu	Arg	Asn	Arg
1490						1495					1500			
Ala	Gln	Asn	Gly	Glu	Val	Pro	Arg	Phe	Lys	Lys	Val	Val	Phe	Arg
1505						1510					1515			
Glu	Phe	Ala	Asp	Gly	Ser	Phe	Thr	Gln	Pro	Ser	Tyr	Arg	Gly	Glu
1520						1525					1530			
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1535						1540					1545			
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1550						1555					1560			
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1580						1585					1590			
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1595						1600					1605			
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1610						1615					1620			
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1625						1630					1635			
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Lys Ser Trp Tyr Phe Thr Glu Asn Val Glu Arg Asn Cys Arg Ala 1670 1675 1680		
Pro Cys His Leu Gln Met Glu Asp Pro Thr Leu Lys Glu Asn Tyr 1685 1690 1695		
Arg Phe His Ala Ile Asn Gly Tyr Val Met Asp Thr Leu Pro Gly 1700 1705 1710		
Leu Val Met Ala Gln Asn Gln Arg Ile Arg Trp Tyr Leu Leu Ser 1715 1720 1725		
Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His 1730 1735 1740		
Val Phe Ser Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Val Tyr 1745 1750 1755		
Asn Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser 1760 1765 1770		
Lys Val Gly Ile Trp Arg Ile Glu Cys Leu Ile Gly Glu His Leu 1775 1780 1785		
Gln Ala Gly Met Ser Thr Thr Phe Leu Val Tyr Ser Lys Glu Cys 1790 1795 1800		
Gln Ala Pro Leu Gly Met Ala Ser Gly Arg Ile Arg Asp Phe Gln 1805 1810 1815		
Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala 1820 1825 1830		
Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Asp 1835 1840 1845		

Pro	His	Ser	Trp	Ile	Lys	Val	Asp	Leu	Leu	Ala	Pro	Met	Ile	Ile
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1865						1870					1875			
Tyr	Ile	Ser	Gln	Phe	Ile	Ile	Met	Tyr	Ser	Leu	Asp	Gly	Arg	Asn
1880						1885					1890			
Trp	Gln	Ser	Tyr	Arg	Gly	Asn	Ser	Thr	Gly	Thr	Leu	Met	Val	Phe
1895						1900					1905			
Phe	Gly	Asn	Val	Asp	Ala	Ser	Gly	Ile	Lys	His	Asn	Ile	Phe	Asn
1910						1915					1920			
Pro	Pro	Ile	Val	Ala	Arg	Tyr	Ile	Arg	Leu	His	Pro	Thr	His	Tyr
1925						1930					1935			
Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu	Met	Gly	Cys	Asp	Leu
1940						1945					1950			
Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Gln	Asn	Lys	Ala	Ile	Ser
1955						1960					1965			
Asp	Ser	Gln	Ile	Thr	Ala	Ser	Ser	His	Leu	Ser	Asn	Ile	Phe	Ala
1970						1975					1980			
Thr	Trp	Ser	Pro	Ser	Gln	Ala	Arg	Leu	His	Leu	Gln	Gly	Arg	Thr
1985						1990					1995			
Asn	Ala	Trp	Arg	Pro	Arg	Val	Ser	Ser	Ala	Glu	Glu	Trp	Leu	Gln
2000						2005					2010			
Val	Asp	Leu	Gln	Lys	Thr	Val	Lys	Val	Thr	Gly	Ile	Thr	Thr	Gln
2015						2020					2025			
Gly	Val	Lys	Ser	Leu	Leu	Ser	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu
2030						2035					2040			
Val	Ser	Ser	Ser	Gln	Asp	Gly	Arg	Arg	Trp	Thr	Leu	Phe	Leu	Gln
2045						2050					2055			

Asp Gly His Thr Lys Val Phe Gln Gly Asn Gln Asp Ser Ser Thr  
2060 2065 2070

Pro Val Val Asn Ala Leu Asp Pro Pro Leu Phe Thr Arg Tyr Leu  
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Arg Ile His Pro Thr Ser Trp Ala Gln His Ile Ala Leu Arg Leu  
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Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr  
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<400> 34

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Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys  
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro  
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val  
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val  
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala  
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val  
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn  
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser  
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu  
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu  
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp  
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser  
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg  
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His  
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu  
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile  
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly  
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met  
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg  
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp  
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe  
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His  
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu  
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro  
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr  
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile  
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile  
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile  
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys  
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys  
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys  
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala  
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp  
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe

565					570					575					
Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu	Asn	Ile	Gln
			580					585					590		
Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	Pro	Glu	Phe
		595					600					605			
Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val	Phe	Asp	Ser
	610					615					620				
Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	Tyr	Ile	Leu
625						630					635				640
Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe	Ser	Gly	Tyr
				645					650					655	
Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr	Leu	Phe	Pro
			660					665					670		
Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	Gly	Leu	Trp
		675					680					685			
Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	Met	Thr	Ala
	690					695					700				
Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	Tyr	Tyr	Glu
705						710					715				720
Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	Asn	Asn	Ala
				725					730					735	
Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Arg	Ser	Thr	Arg
			740					745					750		
Gln	Lys	Gln	Phe	Asn	Ala	Thr	Thr	Ile	Pro	Glu	Asn	Asp	Ile	Glu	Lys
		755					760					765			
Thr	Asp	Pro	Trp	Phe	Ala	His	Arg	Thr	Pro	Met	Pro	Lys	Ile	Gln	Asn
	770					775					780				

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro  
785 790 795 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe  
805 810 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser  
820 825 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val  
835 840 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly  
850 855 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser  
865 870 875 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala  
885 890 895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His  
900 905 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro  
915 920 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp  
930 935 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp  
945 950 955 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys  
965 970 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys  
980 985 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala  
995 1000 1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu  
1010 1015 1020

Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu  
1025 1030 1035

Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp  
1040 1045 1050

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr  
1055 1060 1065

Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly  
1070 1075 1080

Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys  
1085 1090 1095

Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His  
1100 1105 1110

Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln  
1115 1120 1125

Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe  
1130 1135 1140

Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr  
1145 1150 1155

Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn  
1160 1165 1170

Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His  
1175 1180 1185

Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys Lys Glu Thr  
1190 1195 1200

Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr  
1205 1210 1215

Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg  
1220 1225 1230

Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu  
1235 1240 1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys  
1250 1255 1260

His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu  
1265 1270 1275

Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys  
1280 1285 1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr  
1295 1300 1305

Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu  
1310 1315 1320

Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr  
1325 1330 1335

Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr  
1340 1345 1350

Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser  
1355 1360 1365

Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile Pro Gln Ala  
1370 1375 1380

Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser  
1385 1390 1395

Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser  
1400 1405 1410

Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val

1415	1420	1425
Gln Glu Ser Ser His Phe Leu 1430	Gln Gly Ala Lys 1435	Lys Asn Asn Leu 1440
Ser Leu Ala Ile Leu Thr 1445	Glu Met Thr Gly 1450	Asp Gln Arg Glu 1455
Val Gly Ser Leu Gly Thr 1460	Ser Ala Thr Asn Ser 1465	Val Thr Tyr Lys 1470
Lys Val Glu Asn Thr Val 1475	Leu Pro Lys Pro Asp 1480	Leu Pro Lys Thr 1485
Ser Gly Lys Val Glu Leu 1490	Leu Pro Lys Val His 1495	Ile Tyr Gln Lys 1500
Asp Leu Phe Pro Thr Glu 1505	Thr Ser Asn Gly Ser 1510	Pro Gly His Leu 1515
Asp Leu Val Glu Gly Ser 1520	Leu Leu Gln Gly Thr 1525	Glu Gly Ala Ile 1530
Lys Trp Asn Glu Ala Asn 1535	Arg Pro Gly Lys Val 1540	Pro Phe Leu Arg 1545
Val Ala Thr Glu Ser Ser 1550	Ala Lys Thr Pro Ser 1555	Lys Leu Leu Asp 1560
Pro Leu Ala Trp Asp Asn 1565	His Tyr Gly Thr Gln 1570	Ile Pro Lys Glu 1575
Glu Trp Lys Ser Gln Glu 1580	Lys Ser Pro Glu Lys 1585	Thr Ala Phe Lys 1590
Lys Lys Asp Thr Ile Leu 1595	Ser Leu Asn Ala Cys 1600	Glu Ser Asn His 1605
Ala Ile Ala Ala Ile Asn 1610	Glu Gly Gln Asn Lys 1615	Pro Glu Ile Glu 1620

Val	Thr	Trp	Ala	Lys	Gln	Gly	Arg	Thr	Glu	Arg	Leu	Cys	Ser	Gln
1625						1630					1635			
Asn	Pro	Pro	Val	Leu	Lys	Arg	His	Gln	Arg	Glu	Ile	Thr	Arg	Thr
1640						1645					1650			
Thr	Leu	Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile
1655						1660					1665			
Ser	Val	Glu	Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile	Tyr	Asp	Glu	Asp
1670						1675					1680			
Glu	Asn	Gln	Ser	Pro	Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr
1685						1690					1695			
Phe	Ile	Ala	Ala	Val	Glu	Arg	Leu	Trp	Asp	Tyr	Gly	Met	Ser	Ser
1700						1705					1710			
Ser	Pro	His	Val	Leu	Arg	Asn	Arg	Ala	Gln	Ser	Gly	Ser	Val	Pro
1715						1720					1725			
Gln	Phe	Lys	Lys	Val	Val	Phe	Gln	Glu	Phe	Thr	Asp	Gly	Ser	Phe
1730						1735					1740			
Thr	Gln	Pro	Leu	Tyr	Arg	Gly	Glu	Leu	Asn	Glu	His	Leu	Gly	Leu
1745						1750					1755			
Leu	Gly	Pro	Tyr	Ile	Arg	Ala	Glu	Val	Glu	Asp	Asn	Ile	Met	Val
1760						1765					1770			
Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Ser	Phe	Tyr	Ser	Ser
1775						1780					1785			
Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly	Ala	Glu	Pro	Arg
1790						1795					1800			
Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr	Tyr	Phe	Trp	Lys
1805						1810					1815			
Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu	Phe	Asp	Cys	Lys
1820						1825					1830			

Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	Asp	Val	His
1835						1840					1845			
Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Val	Cys	His	Thr	Asn	Thr	Leu
1850						1855					1860			
Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln	Glu	Phe	Ala	Leu
1865						1870					1875			
Phe	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	Tyr	Phe	Thr	Glu
1880						1885					1890			
Asn	Met	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	Met	Glu
1895						1900					1905			
Asp	Pro	Thr	Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly
1910						1915					1920			
Tyr	Ile	Met	Asp	Thr	Leu	Pro	Gly	Leu	Val	Met	Ala	Gln	Asp	Gln
1925						1930					1935			
Arg	Ile	Arg	Trp	Tyr	Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile
1940						1945					1950			
His	Ser	Ile	His	Phe	Ser	Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys
1955						1960					1965			
Glu	Glu	Tyr	Lys	Met	Ala	Leu	Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe
1970						1975					1980			
Glu	Thr	Val	Glu	Met	Leu	Pro	Ser	Lys	Ala	Gly	Ile	Trp	Arg	Val
1985						1990					1995			
Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ala	Gly	Met	Ser	Thr	Leu
2000						2005					2010			
Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	Met	Ala
2015						2020					2025			
Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala	Ser	Gly	Gln	Tyr
2030						2035					2040			

Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	Arg	Leu	His	Tyr	Ser	Gly	Ser
2045						2050					2055			
Ile	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	Trp	Ile	Lys	Val
2060						2065					2070			
Asp	Leu	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	Gly
2075						2080					2085			
Ala	Arg	Gln	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile
2090						2095					2100			
Met	Tyr	Ser	Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn
2105						2110					2115			
Ser	Thr	Gly	Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser
2120						2125					2130			
Gly	Ile	Lys	His	Asn	Ile	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr
2135						2140					2145			
Ile	Arg	Leu	His	Pro	Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg
2150						2155					2160			
Met	Glu	Leu	Met	Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu
2165						2170					2175			
Gly	Met	Glu	Ser	Lys	Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser
2180						2185					2190			
Ser	Tyr	Phe	Thr	Asn	Met	Phe	Ala	Thr	Trp	Ser	Pro	Ser	Lys	Ala
2195						2200					2205			
Arg	Leu	His	Leu	Gln	Gly	Arg	Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val
2210						2215					2220			
Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln	Val	Asp	Phe	Gln	Lys	Thr	Met
2225						2230					2235			
Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly	Val	Lys	Ser	Leu	Leu	Thr

[illegible]

5     **Aventis Behring GmbH**

**2002/M018 (A66)**

**Claims:**

10

1.     Modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment, **characterised in that**

15

A)     one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA are substituted by a different codon in such a way that

20

- when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

25

- when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the opposite charge is introduced into the human sequence,

30

- when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino acid with the opposite charge then a codon for

5 an amino acid with the opposite charge is introduced into the  
human sequence or

10 B) one or several codons for a charged amino acid which are found in the  
FVIII cDNA of a hemophilic patient are replaced by a codon for an amino  
acid of the opposite charge.

2. Recombinant expression vector containing the factor VIII cDNA as claimed in  
claim 1, **characterised in that** it carries in addition transcriptional regulatory  
elements for expression in a suitable host cell.

15

3. Modified biologically active recombinant human factor VIII with improved  
stability wherein mutations are inserted either in the wild-type factor VIII or in a  
factor VIII in which the B-domain is partially or completely deleted and may be  
replaced by a linker, **characterised in that**

20

A.) one or several amino acids of the human factor VIII which are not identical  
with the corresponding amino acid in the same position of the porcine factor  
VIII are substituted by a different amino acid in such a way that

25

- when the human sequence contains a neutral amino acid  
whereas the porcine sequence contains a charged amino acid  
then a charged amino acid with the same charge as found in  
the porcine sequence is introduced into the human sequence;

30

- when the human sequence contains a charged amino acid  
whereas the porcine sequence contains a neutral amino acid  
then a neutral amino acid or an amino acid of the opposite  
charge is introduced into the human sequence;

- 5                   • when the human sequence contains a charged amino acid  
                      whereas the porcine sequence contains an amino acid with the  
                      opposite charge then an amino acid with the opposite charge is  
                      introduced into the human sequence or

10           B)    one or several charged amino acids which are found in the FVIII  
                  amino sequence of hemophilic patients are replaced by a codon for an amino  
                  acid of the opposite charge.

4.       Modified biologically active recombinant human factor VIII as claimed in  
15       claim 3, wherein the plasma half life of its activated form is more than 3 minutes,  
          preferably more than 10 minutes and most preferably more than 30 minutes.

5.       Modified human factor VIII, **characterised in that** its A2-domain is stabilised  
          by the substitution of one or several amino acids as claimed in claim 3.

20

6.       Process for the recombinant production of a modified human factor VIII as  
          claimed in claim 3 either in cell suspension or on a solid support as a bath cell  
          culture or as a perfusion cell culture with continuous production of a conditioned  
          medium **characterised in that** the factor VIII proteins, which are expressed by a  
25       suitable host cell line are purified by chromatographic methods.

7.       Process as claimed in claim 6, **characterised in that** the transcription units  
          encoding the modified factor VIII cDNA of claims 1 and 2 contain a dominant  
          selectable marker in order to facilitate the isolation of specific cell clones which  
30       have integrated said specific c-DNA into their genome.

8.       Host cell line for expression of the factor VIII proteins of claim 3,  
          **characterised in that** it is an animal cell line of vertebrate origin which contains the  
          factor VIII cDNA of claim 1 integrated into its genome.

35

- 5    9.     Pharmaceutical composition, **characterised in that it** comprises a modified  
biologically active recombinant human factor VIII of claim 3.
10.    Vector for gene therapy of hemophilia A, characterized in that it contains  
a modified FVIII cDNA as claimed in claim 1.